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Improved Method for Diagnosis of Polycythemia Vera Based on Flow Cytometric Analysis of Autonomous Growth of Erythroid Precursors in Liquid Culture

Daphna Manor, Eliezer A. Rachmilewitz, and Eitan Fibach*

Department of Hematology, Hadassah University Hospital, Jerusalem, Israel

"Autonomous" development of erythroid colonies in erythropoietin (EPO)-free semi-solid culture has been used as an in vitro assay for diagnosis of polycythemia vera (PV). These colonies, however, are small and poorly hemoglobinized, rendering the assay in many cases unreliable. We report here on the use of a novel assay; it combines a modified culture procedure that maximizes the growth of EPO-independent erythroid cells, and immunofluorescence flow cytometry for their detection and quantitation. Peripheral blood mononuclear cells are cultured for 2–5 days in the presence of a combination of growth factors. During this phase, early erythroid committed progenitors, burst forming units (BFUe), proliferate and differentiate into colony forming units (CFUe)-like progenitors. In the second phase, the latter cells, in the presence of stem cell factor, hemin, and iron-saturated transferrin, continue to proliferate and mature into hemoglobin (Hb)-containing orthochromatic normoblasts. Neither phases contained EPO. The culture produced large, pure, and synchronized erythroid cell populations. The cells were then dually labeled with fluorescent probes, nuclear DNA with thiazole orange and intracellular hemoglobin (Hb) with phycoerythrin-conjugated monoclonal antibodies against human Hb. Cells positive for both labels were assigned as Hb-containing nucleated precursors. The presence of such cells in EPO-free cultures indicated "autonomous growth." None of the EPO-free cultures derived from normal donors or patients with secondary polycythemia contained such cells. Cultures derived from PV patients contained from 5 to 92% "autonomously-grown" cells. These culture and analysis methods should minimize false negative results with PV patients and provide objective and quantitative data. Am. J. Hematol. 54:47–52, 1997

Key words: erythroid cells; erythropoietin; flow cytometry; polycythemia

INTRODUCTION

Polycythemia vera (PV) is characterized by a clonal population of abnormal erythroid progenitors that are supersensitive to EPO [1]. This property has been utilized for laboratory diagnosis of PV; when cells derived from patients with PV are cloned in semi-solid culture medium, erythroid colonies develop in the absence of added EPO [2]. Such "autonomous" colonies are not found in normal cultures nor in cultures derived from other types of polycythemia. This assay, however, is not flawless. "Autonomous" colonies are small and poorly hemoglobinized, and thus hard to be scored and distinguished from clusters of mature RBC which often contaminate the cultures.

We have developed a two-phase liquid culture procedure for growing normal and abnormal erythroid progenitors [3]. In the first phase, peripheral blood mononuclear cells are cultured for 1 week in the presence of a combination of growth factors, but not of EPO. During this phase, early erythroid committed progenitors, burst forming units, proliferate and differentiate into colony forming units. Once becoming EPO-dependent, their proliferation and further differentiation are blocked and they accumulate at a specific stage of differentiation. In the second phase, the latter cells, following exposure to EPO, con-

*Correspondence to: Eitan Fibach, Department of Hematology, Hadassah University Hospital, P.O. Box 12,000, Jerusalem 91120, Israel.

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tinue to proliferate and mature into hemoglobin (Hb)-containing orthochromatic normoblasts.

We report here on the use of the two-phase liquid culture procedure and flow cytometric analysis of autonomously developed erythroid precursors in order to improve on the currently used cloning techniques for in vitro testing of polycythemia.

The liquid culture procedure was modified to maximize to growth of EPO-independent erythroid cells: The first phase was shortened to 2–5 days and EPO was omitted from the second phase, which was supplemented instead with iron-saturated transferrin, hemin, and stem cell factor (SCF). These compounds have been previously shown to increase the yield of erythroid cells in EPO-supplemented liquid cultures [4–6]. Our results indicated that they also have an enhancing effect on PV cells in the absence of EPO.

The analysis of Hb-containing nucleated precursors was assisted by a double labeling flow cytometry. Nuclear DNA was stained with thiazole orange and intracellular Hb by phycoerythrin (PE)-conjugated monoclonal antibodies (MAb). The modified cultures produced large populations of cells for flow cytometry, which in turn provided objective, quantitative, and sensitive analysis.

MATERIALS AND METHODS

Patients

PV patients were selected according to criteria determined by the PV Study Group [7]. All these patients were being managed by phlebotomy at the time of the study.

Cells and Culture Procedure

Peripheral blood (10 to 20 ml) was drawn from normal volunteers and patients with polycythemia, after informed consent was given, in heparin-containing tubes (Becton-Dickinson, Vacutainer Systems, Rutherford, NJ). In some cases, the blood was obtained following phlebotomy, which was performed for clinical reasons. The blood was mixed at a 7:1 ratio (V/V) with Hespan^{R} (6% hetastarch in 0.9% NaCl) (DuPont Pharma, Wilmington, DE). The cells were left to settle down at room temperature for about 1 hr. The upper layer was then collected, layered on Ficoll-Hypaque (density 1.077 g/ml) and centrifuged at 1,000*g* for 20 min at room temperature. The interphase layer was carefully collected, washed three times with phosphate buffered saline (PBS), and seeded at about 5×10^6 cell/ml in alpha minimal essential medium supplemented with antibiotics (50 μg/ml Streptomycin and 50 U/ml Penicillin), 10% preselected fetal calf serum (FCS) (both from GIBCO, Grand Island, NY), 1 μg/ml cyclosporin A (Sandoz, Basel, Switzerland), and 10% conditioned medium collected from cultures of the 5637 bladder-carcinoma cell line. The cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air with extra humidity.

Following 2–5 day incubation in this phase I culture, the nonadherent cells were harvested, washed, and recultured in fresh medium composed of antibiotic-supplemented alpha medium, 30% FCS, 1% deionized bovine serum albumin, 1×10^{-5}M β-mercaptoethanol, 1.5 mM glutamine, 1×10^{-6}M dexamethasone, 0.3 mg/ml iron-saturated transferrin, 0.5 mM hemin, and 10 U/ml human recombinant stem cell factor (SCF) (all from Sigma, St. Louis, MO). For positive control, cultures were supplemented with 1 U/ml human recombinant EPO (Cilag AG, Schauffhausen, Switzerland).

Cells from liquid culture were stained for Hb content by depositing the cells on a glass slide (cytocentrifuge, Shandon, Runcorn, UK), fixing in methanol, and staining with alkaline benzidine-Wright Giemsa reaction. The proportion of nucleated orange-stained cells were scored.

Flow Cytometry

Cells were harvested, washed with PBS, fixed for 30 min with 3% paraformaldehyde and then permeabilized with a solution containing 0.1% saponin and 1% glycine (all purchased from Polysciences Inc. Warrington, PA). The cells were then stained simultaneously with thiazole orange (Aldrich, Milwaukee, WI) at final concentration of 1 μg/ml and PE-conjugated anti HbA mouse MAb (Isolab Inc., Akron, OH) at 1:100 dilution. After 15 min at room temperature the cells were washed and analyzed using FACStar^{plus} flow cytometer (Becton-Dickinson, Immunofluorometry systems, Mountain View, CA). Cells were passed at a rate of 1,000 cells/sec through a 70 μm nozzle, using saline as the sheath fluid. A 488 nm argon laser beam at 250 mW served as the light source for excitation. Green (thiazole orange-derived) fluorescence was measured using a 530 ± 30 nm band-pass filter and red (PE-derived) fluorescence, using a 575 ± 26 nm band filter. The PMTs were set at the appropriate voltage. Logarithmic amplification was applied for measurements of fluorescence and linear amplification for forward light scatter. At least 10^4 cells were analyzed.

RESULTS

Peripheral blood mononuclear cells from PV patients were grown in two-phase cultures as described in Materials and Methods. Hb-containing erythroid precursors first appeared after 5 days in phase II and reached maximal number and maturation after 10–12 days. When EPO was added to phase II, cultures derived from all PV patients studied contained abundant number of erythroid cells. In the absence of EPO, the number of erythroid cells varied widely; cultures derived from some patients contained a high proportion of erythroid cells (as many as 90%), while in others erythroid cells could hardly be observed.

In the former cases the erythroid nature of the cells could be verified by cytochemical benzidine staining, which detects intracellular Hb, while in the latter cases benzidine positive cells were found only after long and tedious microscopic examination.

Addition of iron-saturated transferrin, hemin, and SCF at the onset of phase II increased considerably the number of erythroid cells as well as their Hb content. This was most pronounced in those cases where the autonomous growth was limited (data not shown).

PV cells grown in the absence of EPO were harvested after 10–14 days in phase II, when they reached maximal Hb content, washed, fixed, and labeled with PE-conjugated anti-HbA MAbs and thiazole orange. The results are drawn as two-parameter dot plots (Fig. 1A–D): The ordinates (Fl-2; PE-derived, red fluorescence) indicate intracellular Hb content; Hb-containing cells have bright red fluorescence while cells that do not contain Hb are dull. The abscissa (FL-1; thiazole orange-derived green fluorescence) indicate the presence of nucleated (bright green fluorescent) or non-nucleated (dull green fluorescent) cells. The baselines were set using control cells stained with an isotypic irrelevant antibody (Fig. 1A). Cells stained with thiazole orange only (Fig. 1B) showed $<0.5\%$ overflow of cells into the red-fluorescence zone and cells stained with PE-conjugated anti Hb MAbs (Fig. 1C) showed $<1\%$ overflow of cells into the green fluorescence zone. Cells stained with both PE-conjugated anti-Hb MAbs and thiazole orange (Fig. 1D) showed two clusters of "events" which indicated the presence of two major populations: Hb-containing cells and non Hb-containing cells (non-erythroid cells). Both populations were nucleated, with very few non-nucleated cells (mature RBC). The latter cells were "gated out" and were not included in the analysis.

Figure 1E–H shows two-parameter dot plots of the distribution of normal and PV cells cultured in the presence or absence of EPO. The ordinates (Fl-2; PE-derived red fluorescence) indicate intracellular Hb content, and the abscissa indicate forward light scatter which reflects cell size. Non-nucleated cells ($<1\%$ of total) were gated out. The results show that in normal cultures red fluorescent cells were found in the presence of EPO (Fig. 1F), but not in its absence (Fig. 1E). Thus, verifying the identity of these cells as Hb-containing cells and ascertaining that no hemoglobinized cells developed in normal cultures in the absence of EPO. With PV cells, red fluorescent (erythroid) cells appear in both EPO-supplemented (Fig. 1H) as well as EPO-depleted (Fig. 1G) cultures. The proportion (70 vs. 40%), Hb content (mean fluorescence 386 vs. 276), as well as absolute number (not shown) were much higher in the EPO-supplemented cultures. These results demonstrate the ability of some (abnormal) progenitors derived from PV patients to develop in the absence of EPO, but also indicate that EPO does have a stimulatory effect on PV progenitors. This may be restrictive to the normal progenitors or may involve progenitors derived from the abnormal clone as well.

Table I summarizes the results of cultures derived from normal individuals and polycythemic patients. PV cultures yielded in most cases more than 35% positive cells and no less than 5%, whereas in normal and secondary polycythemia there were no more than 1% positive cells.

DISCUSSION

"Autonomous" colony formation of erythroid progenitors in semi-solid culture medium, in the presence of FCS but the absence of added EPO, has become a useful laboratory tool for diagnosis of PV. In our long experience, which is in agreement with numerous reports by various groups [2,8,9] "autonomous" colonies are specific to PV-derived progenitors and are not found in normal cultures nor in cultures derived from other types of polycythemia. The major technical problem of this assay is not EPO-independent growth of normal cells, but rather poor growth of PV-derived cells under these conditions. "Autonomous" colonies are often small and poorly hemoglobinized and thus sometimes hard to be scored (resulting in false negative results), or difficult to be distinguished from clusters of mature RBC which often contaminate the cultures (resulting in false positive results).

For this reason we selected the most permissive culture conditions for PV erythroid cell growth in EPO-depleted cultures. This included liquid (vs. semi-solid) medium, two-phase (vs. one continuous step) culture, and the addition of stem cell factor (SCF), transferrin, and hemin to phase II. EPO was omitted from both phases of the culture.

Working with normal cells, we have previously shown that the two-phase liquid culture is superior to the semi solid cloning procedure in its erythroid cell yield (up to 5×10^8 per donor), purity (95–98% of the final population are erythroid cells), synchronization with respect to maturation, and the Hb content of the cells [10]. The procedure involves culturing peripheral blood mononuclear cells in liquid medium supplemented, in addition to fetal calf serum, with conditioned medium derived from cultures of the 5637 human bladder carcinoma cell line. This conditioned medium contains a variety of hematopoietic growth factors, but not EPO. During phase I, early erythroid committed progenitors, burst forming units, proliferate and differentiate into late progenitors, colony forming units. Once becoming EPO-dependent, their proliferation and development are blocked and they accumulate at a specific stage of differentiation. The factors present in phase I stimulate, in addition to erythroid progenitors, also myeloid and megakayocyte progenitors. Addition of cyclosporin A inhibits the activation and

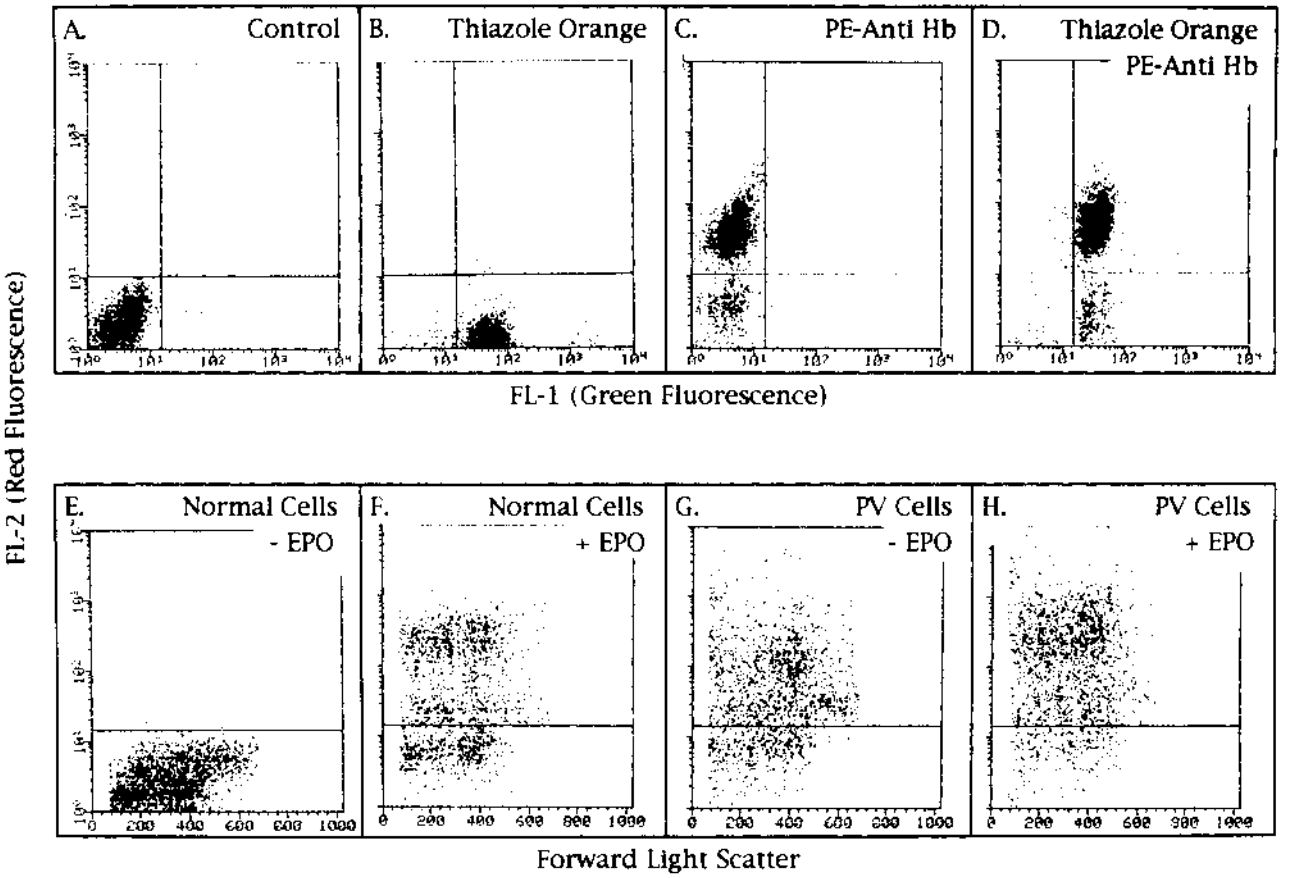

Fig. 1. Flow cytometric analysis of cultured erythroid precursors. Erythroid cells grown in two-phase liquid cultures were harvested on day 12 of phase II and stained with thiazole orange and PE-conjugated anti Hb MAbs (for details see Materials and Methods). A–D: Cells from a PV patient (E.D.) grown in the absence of EPO. The ordinates, Fl-2 (PE-derived, red fluorescence), indicate intracellular Hb content, and the abscissa, FL-1 (thiazole orange-derived green fluorescence), indicates the presence of nucleated or non-nucleated cells. A: Control cells stained with isotypic, irrelevant, MAbs. B: Cells stained with thiazole orange. C: Cells stained with PE-conjugated anti Hb MAbs. D: Cells stained with PE-conjugated anti Hb MAbs followed by thiazole orange. E–H: Normal cells (E, F) or PV cells (G, H) were grown in the absence or presence of EPO and stained with both PE-conjugated anti Hbs and thiazole orange. Non-nucleated cells (<1% of total) were gated out. The ordinates, Fl-2 (PE-derived, red fluorescence), indicate intracellular Hb content and the abscissa indicates forward light scatter, which reflects cell size.

proliferation of lymphoid cells. After 1 week, non-adherent cells are harvested (thus, leaving behind most monocytes and macrophages), washed to remove hematopoietic factors, and recultured in fresh medium supplemented with EPO and high concentration (30%) of preselected fetal calf serum and bovine serum albumin. In phase II, EPO stimulates exclusively erythroid progenitors to further proliferate and mature into Hb-containing orthochromatic normoblasts and some enucleated erythrocytes. Groups of megakaryocytes are also often observed in these cultures. In the absence of the appropriate growth factors, myeloid progenitors largely disappear. Addition of dexamethasone further prevents lymphocyte survival and β-mercaptoethanol improves erythroid cell growth.

In the present study, we have modified the protocol to maximize the growth of EPO-independent PV cells. (1) Most erythrocytes were removed on buoyant density sedimentation with Hespan prior to separation of the mononuclear cells by Ficoll Hypaque. (2) Phase I was shortened to 2–5 days. (3) EPO was omitted from phase II, which was supplemented instead with iron-saturated transferrin, hemin, and stem cell factor (SCF).

The first modification was necessary since PV RBC have light density [see reference 16] and direct separation on Ficoll Hypaque resulted in RBC contamination of the inoculum.

Shortening of phase II resulted in accelerated maturation and more extensive proliferation of the PV precursors as compared to normal precursors. In contrast to normal erythroid progenitors, the development of the EPO-independent PV progenitors is not blocked by the absence of EPO and they proceed in their maturation, uninterrupt-

TABLE I. Flow Cytometric Analysis of Cultured Erythroid Precursors Grown in the Absence of EPO

Diagnosis	Patient	% Positive cells	Patient	% Positive cells
Polycythemia vera	W.S.	47.89	G.A.	10.37
	S.R.	70.76	B.A.	86.84
	D.E.	38.54	N.Y.	92.26
	S.Z.	70.34	M.Y.	70.96
	S.D.	8.72	N.R.	5.17
	O.B.	11.16	D.G.	12.80
	N.A.	6.04	B.N.	5.67
	A.K.	21.44	M.L.	31.73
Secondary polycythemia	G.O.	0.01	D.A.	0.56
	G.S.	0.24	G.D.	0.97
	N.R.	0.86	B.A.	0.45
Normal donors	1	0.01	5	0.68
	2	0.13	6	0.96
	3	0.65	7	0.54
	4	0.88	8	0.60

edly, into the Hb production stage while in phase I. Since the phase I culture medium is not optimally supplemented to support the growth and maturation of erythroid precursors, this phase was shortened and the cells were passed after 2–5 days into phase II.

The inclusion of iron-saturated transferrin, hemin, and SCF have been previously shown to increase the yield of erythroid cells in EPO-supplemented liquid cultures [4–6]. Our present results indicated that they also have an enhancing effect on PV cells in the absence of EPO.

Under EPO-depletion, these supplements caused improved growth of progenitors derived from PV patients, but did not support the growth of progenitors derived from normal individuals or patients with secondary polycythemia. With regard to the effect of SCF, while most authors have shown that SCF worked on normal erythroid progenitors only in synergy with EPO [11–14], Papayannopoulou et al. [15] have reported that some globin synthesizing cells could appear in EPO-depleted one-step cultures of normal $CD34^+$ cells stimulated with both SCF and interleukin-3. But, these globin-producing cells appeared early and died after 6–7 days, never to reach full maturation. In our two-phase procedure, SCF did not induce Hb-synthesis when the cells were cultured without EPO. This might be because we added SCF (as well as hemin and transferrin) only to phase II. Under these conditions, Hb-containing cells first appeared in normal cultures on day 5 of phase II and reached maximum number on day 13, when they were analyzed by flow-cytometry. So that even if early globin-containing cells would have been generated, they would no longer be present at the time of analysis.

To ensure that the Hb-containing cells originated in vitro and were not mature RBC that contaminated the inoculum, the following precautions were taken: (1) Since PV RBC have light density [16] the buffy coat WBC were separated using Hespan^R before they were layered on a Ficoll density gradient. This produced a clean, largely RBC-free, mononuclear cell fraction. (2) The fixation (paraformaldehyde) and permeabilization (saponin) procedures lysed most of the remaining RBC. (3) Staining with thiazole orange permitted "gating out" of non-nucleated cells (RBC), so that the analysis included only nucleated cells.

The use of flow cytometry enabled us to analyze large populations for Hb-containing nucleated cells. In contrast to the cloning techniques, the data thus obtained is objective and quantitative and consequently reproducible and accurate.

The wide range in the absolute numbers and the proportion of positive cells found in cultures of PV patients can be attributed to differences in (1) frequency of erythroid progenitors in the peripheral blood of the patients; (2) proportions of abnormal vs. normal and erythroid- vs. myeloid-committed progenitors; (3) sensitivity to stimulation by growth factors; and (4) proliferation potential in the absence of EPO. These differences could reflect biological evolution of the disease.

With respect to cost effectiveness for diagnosis, the protocol can be divided into two parts: For qualitative protocol, a negative/positive answer by microscopic examination of the phase II liquid cultures would be enough. The liquid culture procedure is not more expensive than the cloning assay and both procedures take about 2 weeks until the cells can be evaluated. Flow eytometric analysis is most helpful in doubtful cases, such as pre-polycythemia state, where full clinical manifestations and laboratory parameters are absent, and when quantitation is required. Although, flow cytometer is quite expensive, it is employed routinely by most hematology laboratories for immunotyping of lymphoma and leukemia cells, and the addition cost involved in staining of the PV cultured cells is not substantial.

In addition, the culture system described here, which

generates pure populations of precursors belonging exclusively to the abnormal PV clone, should be instrumental for molecular studies of the somatic genetic changes that underlie the disease and its evolution.

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